AD685912

Studies on the synthesis of staphylococcal alpha toxin

CHARLES W. HENDRICKS[1,2] AND ROBERT A. ALTENBERN
Biological Sciences Laboratory, Department of the Army, Fort Detrick, Frederick, Maryland
Received May 9, 1968

Several strains of *S. aureus* were induced with mitomycin C, a radiomimetic drug, and the results do not offer direct support for a general explanation for alpha toxin synthesis based upon various aspects of lysogeny. Data are also presented showing that toxin is generated intracellularly and continually released into the medium. Small alkaline changes in pH by the metabolizing culture, not affecting growth, are sufficient to stop or greatly inhibit toxin synthesis. In an actively growing culture, this phenomenon appears as a sharp rise in intracellular toxin synthesis, followed by a peak and then decline, even though the culture continues to grow and increase in optical density. Because the toxin molecule is stable at normal cultural pH values, an alkaline pH may interfere with toxin synthesis at the intracellular level.

Canadian Journal of Microbiology, 14, 1277 (1968)

Certain strains of *Staphylococcus aureus* are capable of producing alpha toxin, which is lethal, dermatonecrotic, and hemolytic to erythrocytes of a variety of animal species (10, 4). It is generally agreed that alpha toxin is a protein with a molecular weight of about 44 000 and is produced only when the culture is undergoing rapid cellular division (8).

Specific mechanisms of alpha toxin induction, synthesis, and subsequent release from the cell are not clear, although Blair and Carr (5) postulated that lysogeny may play a role similar to that described by Barksdale *et al.* (3) for toxinogeny in *Corynebacterium diphtheriae*. The present investigation determined the extent to which lysogeny may be considered as a general explanation for alpha toxinogeny and clarified some of the factors involved in toxin synthesis and release.

Methods

Bacterial Strains

The major portion of this investigation utilized the high alpha-toxin-producing *S. aureus* 233 obtained from Dr. E. D. Rosenblum of the University of Texas, Southwestern Medical School, Dallas, Texas. Eight other alpha-toxin-negative mutants derived from strain 233 were obtained from the same source. Seventeen additional staphylococcal strains, which were originally recovered from clinical material at the Frederick Memorial Hospital Laboratory, were obtained from Fort Detrick sources. Stock cultures of all organisms were maintained on Trypticase soy agar (BBL) slants at 4 °C, and subcultures were made once every 3 months. Before an experiment, the organisms used were cloned on Trypticase soy agar plates containing 5% rabbit blood to obtain a homogeneous population with respect to the toxin characteristics.

Media

The liquid medium utilized in all experiments was essentially that of Coulter (7): Proteose peptone (Difco B120), 15 g per liter; yeast extract (Difco), 5 g per liter; lactic acid (Fisher), 2.5 ml; plus distilled water to make 1 liter. The pH was adjusted to 7.6 with 1 *M* NaOH and the medium autoclaved at 121 °C for 20 minutes. Before use, a small amount of Dow-Corning Antifoam spray was added to each flask.

Trypticase soy broth (Baltimore Biological Laboratories) was employed for initial studies on induction of lysis by mitomycin C.

Alpha Toxin Assay

Culture material for both intra- and extra-cellular alpha toxin assay was prepared by centrifuging (1650 relative centrifugal force (r.c.f.)) 20 ml of the culture for 10 minutes. The resulting supernatant fluid was immediately decanted and titered for alpha toxin by the technique of Cooper *et al.* (6) using twofold dilutions in phosphate-buffered (7.0 pH) saline (0.155 *M* NaCl).

To obtain an estimation of intracellular alpha toxin activity, lysostaphin was added to the sedimented cells to a final concentration of 40 μg per ml, and the suspension was incubated for 1.5 hours at 37 °C. After lysis, the final volume was adjusted to 1.5 ml with buffered saline, and the cellular debris was sedimented by centrifugation (1650 r.c.f.) for 10 minutes. The resulting extract was titered for alpha toxin and assayed for protein by the procedure of Lowry *et al.* (9). The lysostaphin was a gift from Dr. P. A. Tavormina, Mead Johnson Research Center, Evansville, Indiana.

Mitomycin C was obtained from the California Corporation for Biochemical Research. Stock solutions of the drug (0.1 mg per ml) were maintained in 0.1 *M* sodium phosphate buffer (pH 7.4) at 4 °C.

[1]National Academy of Sciences, National Research Council Postdoctoral Research Associate.
[2]Present address: Department of Microbiology, University of Georgia, Athens, Georgia 30601.

Results

Mitomycin C-induced Lysis and Alpha Toxin Synthesis

Preliminary experiments employing cultures in Trypticase soy broth showed that a final concentration of 1.0 μg mitomycin C per ml produced optimal lysis in 2 to 4 hours at 37 °C with sensitive strains. Above this concentration of mitomycin C (2.0 μg per ml or more) growth was totally arrested but no lysis occurred, whereas below 0.5 μg mitomycin C per ml growth was unaffected. This behavior precisely parallels the effects noted during mitomycin C induction of lysis in lysogenic *Escherichia coli* K-12 (12). Of the 17 hospital isolates, 13 strains produced differing amounts of alpha toxin; however, only 5 of the 13 exhibited mitomycin C-induced lysis. Strain 233 and all of the 8 toxin-negative mutants derived from 233 were sensitive to mitomycin C, lysis normally occurring within 2 to 2.5 hours after induction. It should be emphasized that control cultures of the toxin-positive strains were actively producing toxin during the period of induction of lysis by mitomycin C in the cultures so treated; however, in the mitomycin C-treated cultures the titer of α toxin following lysis was no greater than the titer just before the onset of lysis. No effort was made to demonstrate the release of bacteriophage during lysis

Prewarmed 4-liter Erlenmeyer flasks containing 2 liters of unbuffered Coulter's medium were inoculated with cells recovered by centrifugation from an overnight culture of *S. aureus* 233 to yield an initial optical density of 95 Klett units. The flasks were then incubated on a reciprocating shaker at 37 °C in an air atmosphere. Samples were removed at 30-minute intervals and assayed for extracellular and intracellular alpha toxin, total protein, pH, and optical density. When the optical density reached 125 Klett units, the culture was divided into two equal portions; one received mitomycin C (1.0 μg per ml), the other served as the control. Both cultures were returned to the incubator-shaker and assayed periodically as described above. Representative data are presented in Fig. 1. Of considerable interest is the observation that, in the control culture, intracellular toxin appears suddenly and attains nearly maximum concentration within 1 hour. The values plotted for intracellular toxin are specific activity values (units/ml per mg protein) and show that the rate of synthesis of alpha toxin far outstrips the rate of general protein synthesis. In this respect, the phenomenon resembles an induction process. In contrast, extracellular toxin appears somewhat later and exhibits a relatively gradual increase throughout the course of the experiment. It is clear that

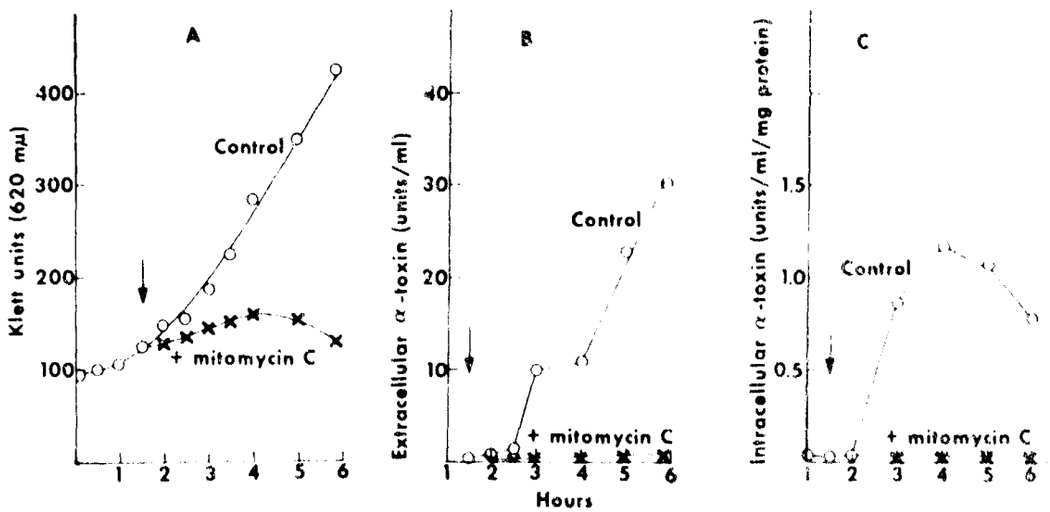

FIG. 1. Mitomycin C induction of lysis of *S. aureus* 233.

mitomycin C exerts a pronounced inhibitory effect on growth and prevents appreciable formation of either intracellular or extracellular alpha toxin.

Medium pH and Alpha Toxin Synthesis

Experiments similar to the foregoing were conducted with cultures in unbuffered Coulter's medium, and the pH of each sample was determined. Typical data from such experiments are presented in Fig 2. These data demonstrate even more clearly the rapid rise of intracellular toxin followed by the later appearance and gradual increase in amount of extracellular alpha toxin. Of considerable pertinence is the fact that the total toxin in the culture (extracellular plus intracellular) continued to rise after the peak intracellular concentration had been attained. In addition, the data strongly suggest that toxin production in unbuffered Coulter's medium is markedly influenced by pH of the culture. When the culture became more alkaline than pH 7.4, toxin synthesis declined rapidly, even though growth proceeded at an unaltered rate

Consequently, experiments were performed in which growing cells of strain 233 that had just initiated toxin production were resuspended in Coulter's medium buffered (phosphate, 0.1 *M*) at specific pH values. The results of these experiments (Fig. 3)

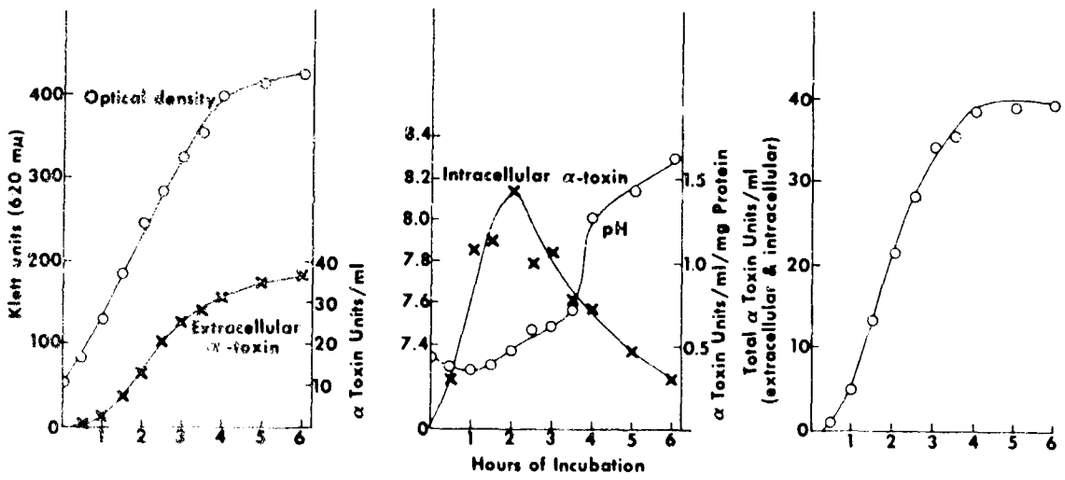

FIG. 2. Alpha toxin production in unbuffered Coulter's medium.

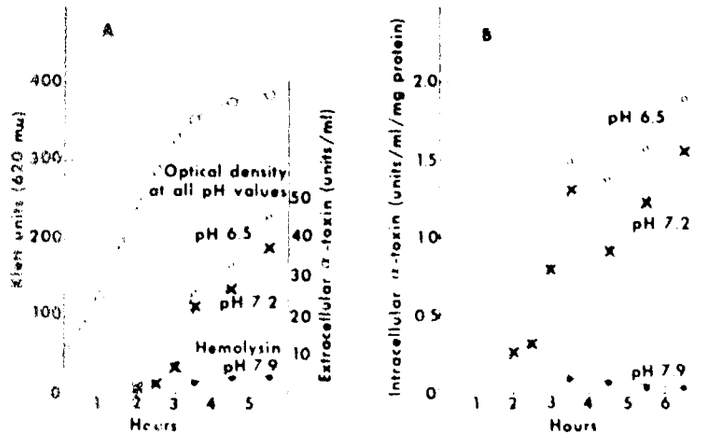

FIG. 3. Alpha toxin production in Coulter's medium adjusted to various pH values. Optical densities were identical at all pH values as indicated in (A).

show that a relatively acidic pH (6.5) permits maximum toxin production. The medium adjusted to pH 7.9 supported only scant toxin production, and intracellular toxin synthesis was almost completely shut off, even though the culture density increased at the same rate as cultures grown in the medium buffered at the lower pH values. These observations were confirmed when Coulter's broth was initially buffered at various pH values and used as the growth medium. Citrate–phosphate buffer was used for pH 4.3, sodium phosphate buffer for pH values of 6.4, 6.9, 7.1, 7.2, 7.4, and 7.8, and Tris buffer for pH values of 8.0 and 8.8. The final concentration of all buffers was 0.1 *M*. No toxin was produced either intracellularly or extracellularly at pH values of 4.3, 8.0, or 8.8, although optical densities were within 20 Klett units of those cultures in which toxin was rapidly produced. A plot of the maximum intracellular toxin concentration attained as a function of the pH of the culture (buffered medium) shows the marked dependence of toxin formation on medium pH (Fig. 4).

In Vitro Complementation

Attempts to demonstrate alpha toxin synthesis by in vitro complementation by both the adjacent-streak technique and mixing cell extracts were without major success. In a few experiments, an increase in titer, always by less than a factor of two, occurred when extracts prepared from the parent strain 233 or one of the mutants were mixed with those from any other mutants. These titers did not increase with time as one might expect, and the small increases observed probably do not represent in vitro complementation.

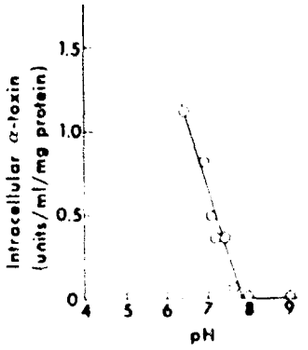

FIG. 4. Alpha toxin production in Coulter's medium buffered to various pH values.

Discussion

Specific mechanisms of alpha toxin synthesis and release have yet to be fully elucidated. It is difficult to justify a general mechanism for alpha toxin synthesis in *S. aureus* based upon lysogeny from the data presented in this study. Not all of the alpha-toxin-producing strains were sensitive to mitomycin C, and the converse was also true. Although the induction of lysis by mitomycin C can offer presumptive evidence of lysogeny, even in the absence of a suitable indicator strain (2), such cursory experiments may not reveal possibly complex relationships between toxigenicity and lysogenic conversion. However, such a phenomenon has been described for the apparent linkage of the loss of beta toxin and gain of fibrinolysin characters in certain strains of *S. aureus* (13). Data in the present study do not support a general synthesis mechanism similar to *C. diphtheriae* (3), where diphtheria toxin is programed by a bacteriophage genome. If prophage induction did play a decisive role in alpha toxin synthesis, a dramatically increased rate of toxin formation should have occurred on the intracellular level when the prophage was induced with mitomycin C. This did not occur, and the total toxin synthesized remained depressed. It is recognized that the possible existence of an inducible but defective prophage or a noninducible prophage controlling α toxin production may invalidate the speculations above.

The surprising behavior of intracellular toxin synthesis towards cultural pH in the uninduced control cultures is of considerable interest. A rapid rise in intracellular toxin was observed in these studies, followed by a peak and then a decline in synthesis, even though the culture continued to grow and increase in optical density. These results show clearly that, at some point in the growth history of the culture, alpha toxin synthesis is switched on or is greatly accelerated. The concept that alpha toxin is synthesized only during a short period followed by a switch

off and external release to the medium can be discounted because the total toxin (intracellular plus extracellular) continues to rise long after the peak intracellular concentration is attained. Mitomycin C induction in Coulter's medium occurred at a pH of approximately 7.3, a value which allowed rapid synthesis of intracellular toxin in uninduced cells.

The sensitivity of staphylococcal products to abrupt or even gradual pH changes during production is not a new concept. A similar observation was made by Altenbern (1) for coagulase, which was not released from growing cells at slightly acidic pH values. Other experiments not presented here indicated that the active toxin molecule is stable to the pH range normally found in a growing culture. Thus, it is likely that toxin synthesis is affected at the intracellular level by pH change, although alkaline conditions might also inhibit the process of release of toxin into the medium.

The data of Coulter (7) and McClatchy and Rosenblum (11) strongly suggested that two cistrons made up the alpha toxin locus. In such a situation, one cistron could be transcribed and translated at an appreciable rate throughout growth, whereas the other cistron could be under very specific pH control. However, in vitro complementation experiments, even with concentrated material, were unsuccessful under a wide variety of conditions, and this particular line of reasoning must be revised.

A great majority of enzymatically active proteins composed of subunits can be readily dissociated into the basic subunits by addition of 8 *M* urea. However, alpha toxin is completely unaffected by urea concentrations as high as 8 *M* or by sulfhydryl reductants such as dithiothreitol (unpublished data). This suggests that the alpha toxin molecule behaves as a single polypeptide chain, not dissociable into smaller units. Consequently, the two transductional groups reported by McClatchy and Rosenblum (11) may be a collection of mutants, each of which bears a mutation at one of two highly mutable points (hot-spots) in the alpha toxin gene.

1. ALTENBERN, R. A. 1966. On the nature of albumin-promoted coagulase release by *Staphylococcus aureus*. J. Infect. Diseases, **116**: 593–600.
2. ALTENBERN, R. A. and STULL, H. B. 1965. Inducible lytic systems in the genus *Bacillus*. J. Gen. Microbiol. **39**: 53–62.
3. BARKSDALE, L., GARMISE, L., and RIVERA, R. 1961. Toxinogeny in *Corynebacterium diphtheriae*. J. Bacteriol. **81**: 527–540.
4. BERNHEIMER, A. W. and SCHWARTZ, L. L. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**: 455–468.
5. BLAIR, J. E. and CARR, M. 1961. Lysogeny in staphylococci. J. Bacteriol. **82**: 984–993.
6. COOPER, L. Z., MADOFF, M. A., and WEINSTEIN, L. 1964. Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. I. Kinetics of the lytic reaction. J. Bacteriol. **87**: 127–135.
7. COULTER, J. R. 1966. Production, purification, and composition of staphylococcal α toxin. J. Bacteriol. **92**: 1655–1662.
8. ELEK, S. D. 1959. *Staphylococcus pyogenes*. E. and S. Livingstone, Ltd., London.
9. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.
10. MADOFF, M. A. and WEINSTEIN, L. 1962. Purification of staphylococcal alpha-hemolysin. J. Bacteriol. **83**: 914–918.
11. MCCLATCHY, J. K. and ROSENBLUM, E. D. 1966. Genetic recombination between α-toxin mutants of *Staphylococcus aureus*. J. Bacteriol. **92**: 580–583.
12. OTSUGI, N., SEKIGUCHI, M., IIJIMO, and TAKAGI, Y. 1959. Induction of phage formation in the lysogenic *Escherichia coli* K12 by mitomycin C. Nature, **184**: 1079.
13. WINKLER, K. C., DE WAART, J., and GROOTSEN, C. 1965. Lysogenic conversion of staphylococci to loss of β-toxin. J. Gen. Microbiol. **39**: 321–333.